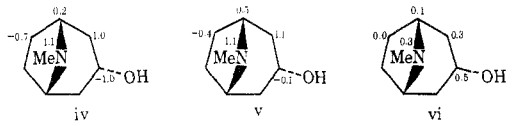
methanol solutions. The $\Delta\delta$ values [$\delta(-30°) - \delta(29°)$] for the two solutions are portrayed on formulas iv and v, respectively. A threefold dilu-



iv v vi

tion of a 2 *M* deuteriochloroform solution which had yielded the shifts denoted on formula **5** leads to the $\Delta\delta$ values [δ(dil) − δ(concd)] illustrated on formula vi.

(13) Curiously, the introduction of the benzoyloxy group at C(6) shields C(2) and C(4), the latter twice as much as the former. Since the 6-oxy substituent is γ-equatorially oriented to C(4) within the cycloheptane nucleus, part of its shielding may be due to the effect recently noted in six-membered ring systems.[14]

(14) E. L. Eliel, W. F. Bailey, L. D. Kopp, R. L. Willer, D. M. Grant, R. Bertrand, K. A. Christensen, D. K. Dalling, W. M. Duch, E. Wenkert, F. M. Schell, and D. W. Cochran, *J. Am. Chem. Soc.*, **97**, 322 (1975).

(15) The shift alterations in the pyrrolidine ring on introduction of a 6β-hydroxy group (**1c** → **2c**) can be used to confirm the pyrrolidine carbon shifts in a 7β-hydroxy compound (**3b**). The $\Delta\delta$(**2c** − **1c**) values for C(5), C(6), C(7), and C(1) are 7.4, 50.9, 15.3, and −1.0 ppm, respectively. Their sequential application to C(1), C(7), C(6), and C(5) of **3b** yields the theoretical values of 71.2, 72.7, 40.6, and 59.0 ppm, respectively, in close agreement with the found shifts.

Sulfur-Containing Polypeptides. XVIII. Unambiguous Synthesis of the Parallel and Antiparallel Isomers of Some Bis-Cystine Peptides[1-3]

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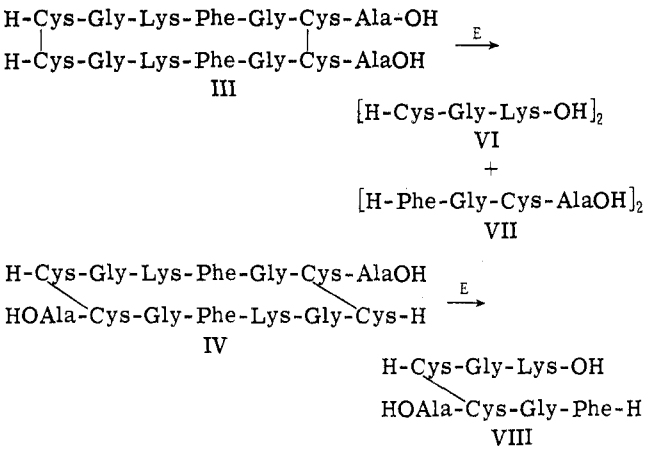
The synthesis of the parallel (III) and antiparallel (IV) isomers and cyclic monomer (V) of the L-cystylglycyl-L-lysyl-L-phenylalanylglycyl-L-cystyl-L-alanine system via the thiocyanogen–sulfenyl thiocyanate method is described. Enzymic degradation of III and IV using trypsin and α-chymotrypsin were employed to establish the pairing of the cystine residues. The thiocyanogen–sulfenyl thiocyanate method has been shown to proceed without intermediate disulfide interchange.

As part of a program directed toward the development of methods for the laboratory synthesis of polypeptides containing several cystine residues,[1] unequivocal methods for the stepwise and selective conversion of various S-protected cysteine thiols to cystine residues with the desired sulfur pairing have been studied. A route which indicated some promise[4] has been the utilization of *S*-trityl and *S*-benzhydryl thioethers of cysteine and subsequent selective oxidative removal of these protective groups by thiocyanogen or sulfenyl thiocyanates of cysteine, the former group being removed without catalysis, the latter requiring acidic conditions.

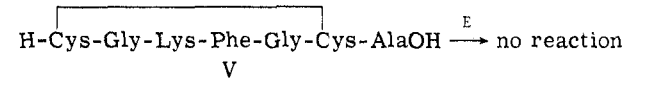
Unfortunately, the isomeric parallel (I) and antiparallel (II) bis-cystine dimers prepared by the sulfenyl thiocyanate method exhibited virtually identical physical properties and appeared to differ only in the magnitude of their optical rotations (the parallel dimer of the L-peptide having the greater negative rotation). Furthermore, the parallel and antiparallel dimers of a particular series could not be distinguished by thin layer or column chromatography.[4,5] In view of the possibility that the acid conditions[4] required for oxidative removal of the *S*-benzhydryl groups (or a thiol–disulfide interchange process as shown in Scheme I) could in fact lead to equilibration of the dimers, which could not be distinguished analytically, a bis-cystine system in which the purity of the isomeric dimers could be unequivocally established was developed.

The peptides of choice were the molecules III–V; treatment of III with trypsin should yield two cleavage products (VI, VII). Similar treatment of IV with trypsin would afford only VIII. Enzymic digestion with α-chymotrypsin should lead to a similar situation with cleavage occurring at the amide bond between Phe-Gly in both III and IV.

Relatively little is known of the parameters which effect enzymic cleavage of cystine containing peptides. Schally and Barrett[6] demonstrated that the antiparallel dimer of [Lys^8] vasopressin (40-membered ring) was cleaved at the



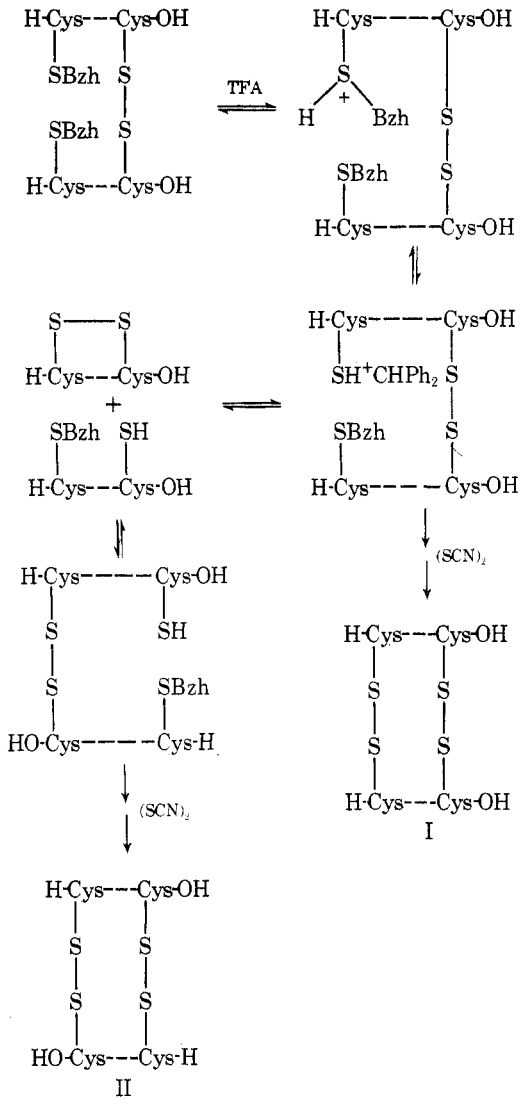
Phe-Gln bond by α-chymotrypsin. Walter and Hoffman[7] showed that oxytocin, lysine vasopressin, and arginine vasopressin (20-membered rings) were resistant to the action of α-chymotrypsin at an enzyme–substrate ratio of 1:300, whereas the corresponding S-alkylated nonapeptides were smoothly cleaved by the enzyme. The cyclic pentapeptide cyclo(Gly-Lys-Gly-Lys-Gly) is resistant to trypsin hydrolysis (15-membered ring) although the linear system is cleaved.[8] The cyclic monomer V (20-membered ring) was therefore expected to be inert to either the action of trypsin or α-chymotrypsin. If these expectations were realized III,



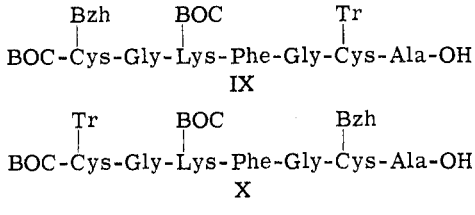
IV, and V could be distinguished from one another (since cyclic monomers have different TLC mobilities than bis dimers) and the specificity of the thiocyanogen–sulfenyl thiocyanate reaction could be established.

The heptapeptide derivatives required for the synthesis

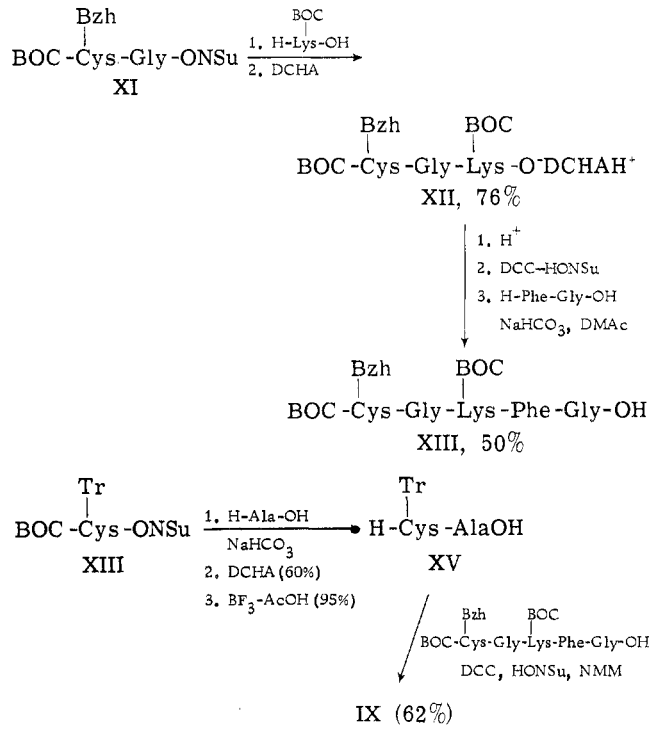
Scheme I



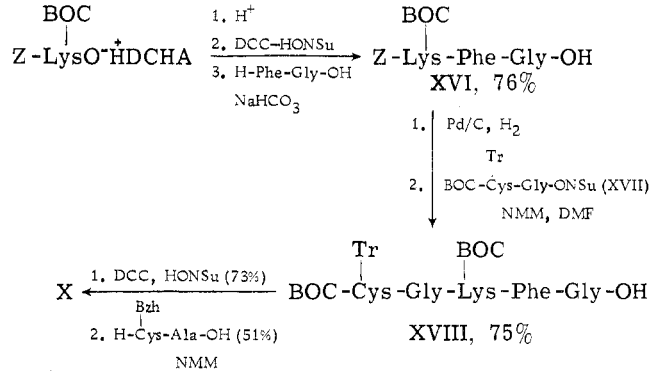
of III–V were *N-tert*-butyloxycarbonyl-*S*-benzhydryl-L-cysteinylglycyl-*N*$^{\epsilon}$-*tert*-butyloxycarbonyl-L-lysyl-L-phenylalanylglycyl-*S*-trityl-L-cysteinyl-L-alanine (IX) and the isomeric peptide derivative, X. The route developed for the



synthesis of the N-terminal pentapeptide portion of IX involved the conversion of XI to XIII. The protected dipeptide required for the elongation of XIII to IX was obtained in low yield (40%) by the DCC coupling of *N,S*-ditrityl-L-cysteine and *tert*-butyl L-alaninate; the preparation was always contaminated with *N*-acylurea and unreacted ester and was difficult to purify owing to the similarity in solubility between reactants and products. These difficulties could be circumvented by preparation of *N-tert*-butyloxycarbonyl-*S*-trityl-L-cysteinyl-L-alanine (XIV) and conversion of this substance to the free base, *S*-trityl-L-cysteinyl-L-alanine (XV). The coupling reaction between the crude *N*-hydroxysuccinimide ester of XIII and XV proceeded smoothly to provide IX in 62% yield.



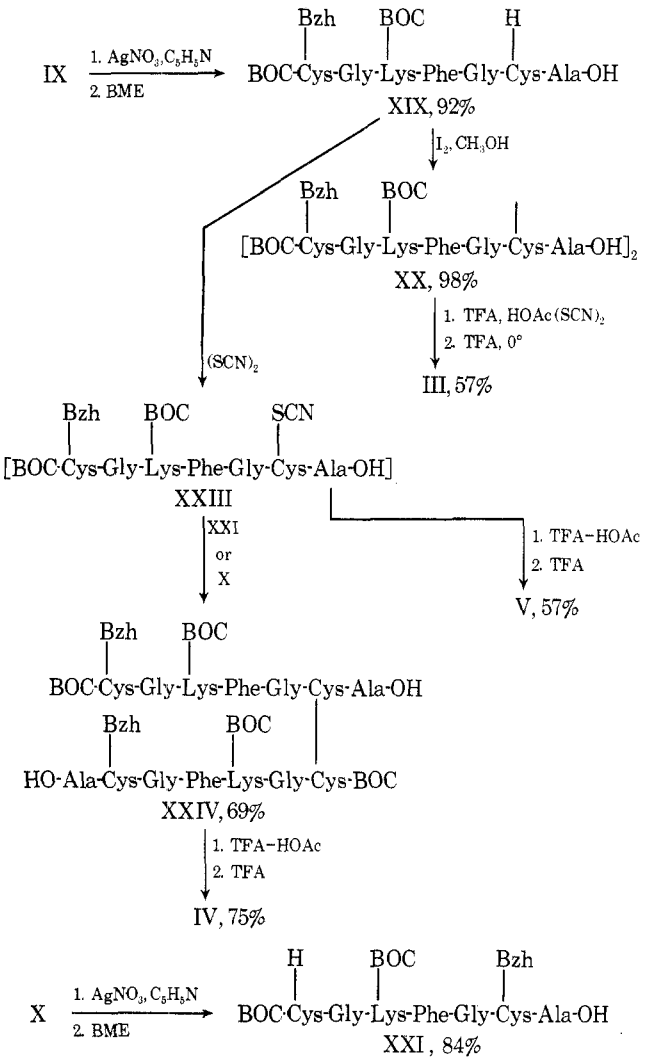
The synthesis of the isomeric heptapeptide derivative, X, was initially attempted via coupling of the *N*-hydroxysuccinimide ester of *N-tert*-butyloxycarbonyl-*S*-trityl-L-cysteinylglycine (XVII) and ϵ-*tert*-butyloxycarbonyl-L-lysine. The reaction did not proceed cleanly and the desired tripeptide was invariably contaminated with unreacted dipeptide; similar results were obtained with the mixed anhydride method of coupling. These problems likely reflected the solubility differences of the reactants. The successful synthetic route involved the formation of *N*-carbobenzyloxy-*N*$^{\epsilon}$-*tert*-butyloxycarbonyl-L-lysyl-L-phenylalanylglycine (XVI). Removal of the *N*-carbobenzyloxy group fol-



lowed by coupling with the *N*-hydroxysuccinimide ester (XVII) provided the protected pentapeptide (XVIII) in 75% yield. Conversion of XVIII to the *N*-hydroxysuccinimide active ester followed by coupling with *S*-benzhydryl-L-cysteinyl-L-alanine provided the desired heptapeptide derivative, X.

The preparation of the required bis-cystine dimers III and IV was achieved by the thiocyanogen method (Scheme II). Treatment of IX with a solution of silver nitrate in pyridine[9,10] allowed selective removal of the *S*-trityl group and afforded the thiol XIX in high yield; oxidation of XIX with iodine provided the disulfide XX. The heptapeptide X was converted to the thiol XXI in the same manner. Iodine oxidation of XXI provided the isomeric disulfide XXII. Treatment of XX (5×10^{-4} *M*) with freshly prepared thiocyanogen solution at 0° provided the crude parallel bis dimer III

Scheme II



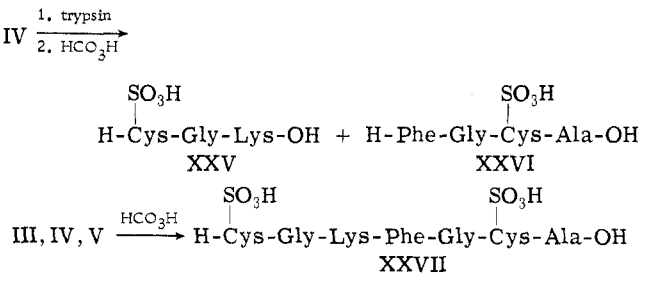
from which the BOC groups had been partially removed. Complete removal of the BOC groups was accomplished by the action of trifluoroacetic acid at 0°. The crude dimer preparation indicated no contamination by cyclic monomer V; however, the preparation invariably contained bound metal ions (probably calcium) as indicated by a residue after elemental analysis. In an attempt to minimize metal ion incorporation all solvents used for thiocyanogen generation were freshly distilled and glassware was carefully cleaned. The resulting crude parallel bis dimer preparation was chromatographed on Bio-Rex-70, a weak cation resin, with a linear gradient of aqueous acetic acid (1–80%). Elemental analysis of the first peak eluted indicated the presence of fluoride ion and inorganic residue. Amino acid and elemental analysis of this fraction were consistent with a monocalcium tetratrifluoroacetate salt of III. The second peak eluted (57%) was homogeneous on TLC and electrophoresis, did not contain metal ions, and gave an acceptable amino acid analysis for the desired parallel dimer, III.

The cyclic monomer V was prepared via the sulfenyl thiocyanate XXIII, generated by addition of XIX in acetic acid to thiocyanogen in ethyl acetate at 0°. A mixture of trifluoroacetic acid–acetic acid (1:3 v/v) was added to the reaction mixture; a concentration of 1.1×10^{-3} *M* was used to maximize intramolecular cyclization. The amino protective groups were completely removed with TFA at 0° and the crude product was purified on Bio-Rex-70. The cyclic monomer V was homogeneous on TLC and electrophoresis and could be distinguished from IX on TLC; no inorganic residue was observed in any preparation of V.

Preparation of the antiparallel bis-cystine dimer IV required the initial formation of the unsymmetrical cystine derivative XXIV. The conditions finally adopted depended on maximizing the solubility of the reactants; preparation of the thiocyanogen solution in chloroform rather than ethyl acetate improved the solubility of the reaction mixture. The thiol XIX in a mixture of acetic acid–chloroform was added to a solution of thiocyanogen in chloroform to generate the sulfenyl thiocyanate XXIII. Treatment of XXIII with either the *S*-trityl heptapeptide X or the corresponding thiol XXI provided the unsymmetrical disulfide XXIV. The material was contaminated with small amounts of unreacted starting materials, symmetrical disulfide XX, and thiocyanogen polymer; these could be removed by recrystallization to yield XXIV, homogeneous by TLC. The conversion of XXIV to IV was achieved as previously described. Chromatography on Bio-Rex-70 indicated a small amount of tetratrifluoroacetate salt of IV containing calcium ion, and a major peak (75%) corresponding to a residue-free sample of IV. The parallel and antiparallel bis-cystine dimers III and IV exhibited identical mobilities on TLC and paper electrophoresis; the parallel dimer exhibited a specific rotation of greater negative magnitude than that of the antiparallel isomer ($[\alpha]^{25}D$ −62.3, −46.4°, respectively) as previously observed.[4]

The conversion of bis-cystine peptides to the corresponding cyclic monomers under alkaline conditions has been previously reported.[4] A preliminary study indicated that III and IV were stable at pH 7.1 for at least 24 hr and that base-catalyzed disulfide interchange leading from III or IV to V would be negligible under these conditions. Hydrolysis of III with trypsin was performed at pH 7.1 and the reaction mixture was analyzed by paper electrophoresis. The tryptic digest exhibited two peptide fragments well separated by electrophoresis; no unreacted III was observed and subsequent studies showed that under these conditions (enzyme–substrate ratio 1:20 w/w) III was completely hydrolyzed in 5 min. Preparative paper electrophoresis, elution of the two spots, and amino acid analysis of each of the oxidized peptide acid hydrolysates indicated that the two open-chain symmetrical cystine peptides VI and VII were produced.

The tryptic hydrolysis of the antiparallel bis-cystine peptide IV was performed under the same conditions. Paper electrophoresis of the reaction mixture indicated that only one product, ultimately shown to be the unsymmetrical open-chain cystine peptide VIII, was produced. The electrophoretic migration distance of the bis dimer IV and the peptide VIII were similar and additional evidence for the identity of VIII was desirable. The peptides IV and

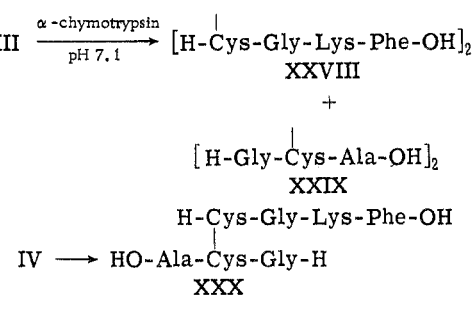


VIII could be distinguished by performic acid oxidation of the tryptic hydrolysis mixture resulting from IV; two ninhydrin positive products were observed corresponding to XXV and XXVI. A spot corresponding to XXVII produced by independent oxidations of III–V was not observed. Thus

the complete tryptic conversion of IV to VIII with no trace of VI or VII was established.

The cyclic monomer V was unaffected by the action of trypsin at pH 7.1 for 2 hr, although prolonged digestion or digestion at pH 8.6 produced some cleavage and a spot corresponding to VIII could be observed. The stability of cyclic cystine derivatives of this type to trypsin and chymotrypsin has been previously noted.[7]

The peptides III–V were also cleaved with α-chymotrypsin at substrate–enzyme ratios of 50:1 (w/w). The results of this study were similar to those obtained with trypsin. Digestion of III with α-chymotrypsin produced the peptides XXVIII and XXIX. Similar treatment of the antiparallel isomer IV provided only XXX. The open-chain unsymmetrical cystine derivative XXX could be distinguished from IV by performic acid oxidation of the enzymic digest. The cyclic monomer was inert to the enzyme at pH 7.1 but was slowly hydrolyzed at pH 8.6.



These experiments establish the purity of the parallel and antiparallel bis-cystine peptides III and IV. In addition these degradations provide independent support for the structural assignments of III and IV and hence for the reliability of the use of thiocyanogen and sulfenyl thiocyanates for the stepwise synthesis of peptides containing several cystine residues.

Experimental Section

General. Melting points were measured on a Thomas-Hoover melting point apparatus and are uncorrected. Elemental analyses were performed by Atlantic Microlab, Atlanta, Ga., and Micro-Tech Laboratories, Skokie, Ill. Controlled pH reactions were operated with a Radiometer pH titrator and magnetic valve. Optical rotations were measured with a Perkin-Elmer Model 141 polarimeter. Amino acid analyses were performed on a Beckman Model 116 unit.

Trypsin (bovine pancreas) was obtained from Sigma Chemical Co.; α-chymotrypsin (bovine pancreas) was the product of Worthington Biochemical Corp. Bio-Rex 70 was purchased from Bio-Rad Laboratories.

Thin layer chromatograms were performed on microscope slides or 5 × 20 cm plates uniformly coated with silica gel GF-254. Spots were visualized with iodine vapor or a spray reagent of ninhydrin. The following solvent systems were used: chloroform–methanol (9:1, system A); chloroform–methanol (19:1, system B); chloroform–methanol–acetic acid (8:1:1, system C); chloroform–methanol–acetic acid (18:1:1, system D); chloroform–methanol–28% ammonia (24:6:1, system E); chloroform–methanol–28% ammonia (12:6:1, system F); 1-butanol–acetic acid–H_2O (4:1:5, system G); *tert*-butyl alcohol–1-butanol–H_2O (4:3:3, system H); 2-butanone–acetic acid–H_2O (6:1:3, system I).

Electrophoresis was performed with a Gilson high voltage electrophorator Model D on Whatman No. 3 MM chromatograph paper (46 × 57 cm). Chromatography was carried out in a pH 3.7 buffer solvent system (pyridine–acetic acid–water, 1:10:289 v/v) at 3000 V (110 mA) from 60 to 80 min. The paper was allowed to dry in air or in the oven (80°) for 15 min, and spots were visualized with ninhydrin (0.2% in acetone) spray reagent.

***N*-*tert*-Butyloxycarbonyl-*S*-benzhydryl-L-cysteinylglycyl-N^{ϵ}-*tert*-butyloxycarbonyl-L-lysine *N,N*-Dicyclohexylammonium Salt (XII).** A mixture of 3.2 g (13 mmol) of N^{ϵ}-*tert*-butyloxycarbonyl-L-lysine[11] and 1.82 g (13 mmol) of potassium carbonate in 2.5 ml of water was treated with a solution containing 5.5 g (10 mmol) of XI[12] in 2.5 ml of DME. The reaction mixture was stirred for 2 hr and neutralized with 20% acetic acid, and the solvent was removed in vacuo. The oil was extracted with ethyl acetate and the organic layer was washed with water and saturated brine. Removal of the solvent and treatment of an ether solution of the foam with *N,N*-dicyclohexylamine (4 ml, 20 mmol) provided a crystalline salt, recrystallized from methanol–ether: 6.48 g (76%); mp 107–110°; $[\alpha]^{23}D$ −9.93° (*c* 1, DMAc); homogeneous (system C).

Anal. Calcd for $C_{46}H_{71}N_5O_8S$: C, 64.68; H, 8.38; N, 8.20; S, 3.75. Found: C, 64.43; H, 8.27; N 7.86; S, 3.73.

***N*-*tert*-Butyloxycarbonyl-*S*-benzhydryl-L-cysteinylglycyl-N^{ϵ}-*tert*-butyloxycarbonyl-L-lysyl-L-phenylalanylglycine (XIII).** A solution of the oil obtained by neutralization of 2.1 g (2.5 mmol) of XII and 0.322 g (2.8 mmol) of *N*-hydroxysuccinimide in 10 ml of DME was cooled to −10° and treated with 0.53 g (2.8 mmol) of DCC. The solution was stirred for 12 hr, the DCU removed, and the solvent evaporated. The resulting oil was dissolved in 5 ml of DMAc and added to a solution containing 0.63 g (2.6 mmol) of L-phenylalanylglycine[12] and 0.44 g (5.2 mmol) of sodium bicarbonate in 5 ml of water. The reaction mixture was stirred for 12 hr, neutralized with 1 *N* sulfuric acid, and extracted with chloroform. The extract was washed with 1 *N* sulfuric acid, water, and brine and dried. Removal of the solvent and addition of ether provided a white solid which was crystallized from acetone to provide 1.03 g (50%) of the pentapeptide derivative: mp 165–167°; $[\alpha]^{23}D$ −25.0° (*c* 1.03, DMAc); homogeneous (system D).

Anal. Calcd for $C_{45}H_{60}N_6O_{10}S$: C, 61.62; H, 6.90; N, 9.58; S, 3.65. Found: C, 61.55; H, 6.97; N, 9.62; S, 3.55.

Amino acid analysis of an acid hydrolysate without performic acid oxidation: $Gly_{2.0}$, $Phe_{1.0}$, $Lys_{1.06}$.

***N*-*tert*-Butyloxycarbonyl-*S*-trityl-L-cysteinyl-L-alanine *N,N*-Dicyclohexylammonium Salt (XIV).** A solution of the oil obtained from neutralization of 12.56 g (20 mmol) of *N*-*tert*-butyloxycarbonyl-*S*-trityl-L-cysteine *N,N*-dicyclohexylammonium salt[12] and 2.76 g (24 mmol) of *N*-hydroxysuccinimide in 30 ml of DME at −10° was treated with 5 g (24 mmol) of DCC. The reaction mixture was stirred for 2 hr at −10° and overnight at room temperature. The DCU was removed and the solvent was evaporated to yield a foam which was dissolved in ethyl acetate and washed with saturated sodium bicarbonate, water, and saturated brine. The solvent was removed and the resulting oil was dissolved in DME and treated with a solution containing 2.23 g (25 mmol) of L-alanine and 5.1 g (50 mmol) of potassium bicarbonate in 50 ml of water. After 4 hr the pH was adjusted to 3.0 with 1 *N* sulfuric acid and the precipitated solid was extracted into ethyl acetate. The solution was washed with water and saturated brine, dried, and evaporated to a foam. The foam was dissolved in 30 ml of ether and treated with 6.0 ml (30 mmol) of *N,N*-dicyclohexylamine to yield a crystalline solid (7.92 g, 60%): mp 153–154°; $[\alpha]^{26}D$ +15.4° (*c* 1, DMF); hydrogeneous (system C).

Anal. Calcd for $C_{42}H_{57}N_3O_5S$: C, 70.44; H, 8.01; N, 5.86; S, 4.47. Found: C, 70.20; H, 8.04; N, 5.86; S, 4.57.

***S*-Trityl-L-cysteinyl-L-alanine (XV).** A solution containing 1.372 g (2 mmol) of the above salt in 10 ml of glacial acetic acid was treated with 0.87 ml (6 mmol) of boron trifluoride etherate. The solution was stirred for 1 hr at room temperature and poured into a solution of 5 g of sodium acetate in 70 ml of water. The white solid was collected and washed with water and ether to yield 0.897 g (95%) of XV: mp 216–218°; $[\alpha]^{25}D$ −2.35° (*c* 0.98, DMF); homogeneous (system C, E).

Anal. Calcd for $C_{25}H_{26}N_2O_3S \cdot 2H_2O$: C, 63.70; H, 6.40; N, 5.91; S, 6.80. Found: C, 64.55; H, 6.37; N, 5.90; S, 6.90.

***N*-*tert*-Butyloxycarbonyl-*S*-benzhydryl-L-cysteinylglycyl-N^{ϵ}-*tert*-butyloxycarbonyl-L-lysyl-L-phenylalanylglycyl-*S*-trityl-L-cysteinyl-L-alanine (IX).** To a solution of 2.17 g (2.48 mmol) of XIII and 0.862 g (7.5 mmol) of *N*-hydroxysuccinimide in 10 ml of DMF at −10° was added 0.612 g (3 mmol) of DCC. The reaction mixture was stirred for 2 hr at −10° and overnight at 0°. After removal of DCU the filtrate was poured into 400 ml of ether. The precipitate was suspended in hot 2-propanol, cooled to room temperature, and collected. The active ester (2.0 g, 85%) was used without further purification.

The solution of the active ester in 20 ml of DMF was added to a solution containing 1.04 g (2.4 mmol) of XV and 0.25 g (2.4 mmol) of *N*-methylmorpholine in 8 ml of DMF. The solution was stirred for 24 hr and poured into cold 1 *N* sulfuric acid (500 ml), and the white solid was collected. The solid was washed with 1 *N* sulfuric acid and water and dried. The white powder was stirred in ether, filtered, and precipitated two times from acetic acid by addition of ether. Recrystallization from 2-propanol provided 1.61 g (62%) of

white powder IX: mp 189–191° dec; $[\alpha]^{25}D$ −26.89° (c 0.5, DMF); homogeneous (system D).

Anal. Calcd for $C_{70}H_{84}N_8O_{12}S_2$: C, 65.02; H, 6.55; N, 8.67; S, 4.96. Found: C, 64.89; H, 6.63; N, 8.61; S, 5.03.

The amino acid analysis of a performic acid oxidized acid hydrolysate was: $CySO_3H_{1.74}$, $Gly_{2.0}$, $Ala_{0.91}$, $Phe_{1.3}$, $Lys_{0.97}$.

N^{α}-Carbobenzyloxy-N^{ϵ}-*tert*-butyoxycarbonyl-L-lysyl-L-phenylalanylglycine (XVI). A solution of N^{α}-carbobenzyloxy-N^{ϵ}-*tert*-butyloxycarbonyl-L-lysine *N*-hydroxysuccinimide ester[13] (2.77 g, 5.8 mol) in DME (25 ml) was added to L-phenylalanylglycine (1.81 g, 7.54 mmol) and sodium bicarbonate (1.35 g, 16 mmol) in water (20 ml). The reaction mixture was stirred for 24 hr, acidified with cold 1 *N* sulfuric acid, and extracted with ethyl acetate (200 ml). The extract was washed with water and saturated brine, dried over magnesium sulfate, filtered, and evaporated to a white solid. The crude compound was stirred with ether and filtered three times to yield 2.56 g (76%) of product: mp 120–123°; $[\alpha]^{26}D$ −32.7° (c 0.505, MeOH); homogeneous (system F, G).

Anal. Calcd for $C_{30}H_{40}N_4O_8$: C, 61.62; H, 6.89; N, 9.58. Found: C, 61.61; H, 6.94; N, 9.54.

N^{ϵ}-*tert*-Butyloxycarbonyl-L-lysyl-L-phenylalanylglycine was prepared by passing hydrogen gas through a mixture of XVI (2.56 g, 4.38 mmol) and glacial acetic acid (3 ml) in methanol (30 ml) in the presence of 0.3 g of 10% palladium on charcoal catalyst. After 4 hr, the solution was filtered and evaporated to a solid which was dissolved in methanol and precipitated by the addition of ether. The precipitate was collected and yielded 1.94 g (99%) of solid: mp 181–184° dec; $[\alpha]^{26}D$ +21.2° (c 0.5, HOAc); homogeneous (system G).

Anal. Calcd for $C_{22}H_{34}N_4O_6$: C, 58.64; H, 7.61; N, 12.43. Found: C, 58.77; H, 7.66; N, 12.29.

N-*tert*-Butyloxycarbonyl-S-trityl-L-cysteinylglycyl-N^{ϵ}-*tert*-butyloxycarbonyl-L-lysyl-L-phenylalanylglycine (XVIII). A solution of XVII (463.5 mg, 0.75 mmol) in DMF (1 ml) was added to a mixture of N^{ϵ}-*tert*-butyloxycarbonyl-L-lysylphenylalanylglycine (225.3 mg, 0.5 mmol) and *N*-methylmorpholine (56 mg, 0.5 mmol) in DMF (3 ml). The reaction mixture was stirred for 24 hr and poured into ether (100 ml). The white precipitate was collected by filtration, washed with 1 *N* sulfuric acid and water, and dried in vacuo. Two crystallizations from 2-propanol gave a solid (450 mg). The solid was dissolved in DMF (2 ml) and applied to a silica gel (40 g) column in chloroform. Elution with 19:1 (v/v) chloroform–methanol removed the DMF and unreacted dipeptide XVI as the first fractions. Further elution provided the product XVIII. Two recrystallizations of the material from methanol–ether afforded a white solid (362 mg, 77%): mp 180–181° dec; $[\alpha]^{25}D$ −5.58° (c 0.555, DMF); homogeneous (system D).

Anal. Calcd for $C_{51}H_{64}N_6O_{10}S$: C, 64.25; H, 6.77; N, 8.81; S, 3.36. Found: C, 64.17; H, 6.92; N, 8.70; S-3.45.

N-*tert*-Butyloxycarbonyl-S-benzhydryl-L-cysteinyl-L-alanine N,N-Dicyclohexylammonium Salt. A solution of the oil obtained from the neutralization of 22.76 g (40 mmol) of N-*tert*-butyloxycarbonyl-S-benzhydryl-L-cysteine *N,N*-dicyclohexylammonium salt and 5.52 g (48 mmol) of *N*-hydroxysuccinimide in 40 ml of DME was cooled to −10° and treated with 9.9 g (48 mmol) of DCC. The reaction mixture was stirred for 2 hr at −10° and overnight at room temperature. DCU was removed by filtration and the remaining solution was evaporated to a foam which was dissolved in ether and washed with saturated sodium bicarbonate, water, and brine, dried over magnesium sulfate, filtered, and evaporated to a foam. The foam was dissolved in 100 ml of DME and added to a solution of 4.46 g (50 mmol) of L-alanine and 10.2 g (100 mmol) of potassium bicarbonate in 100 ml of water. After 17 hr, the pH was adjusted to 3 with 1 *N* sulfuric acid and the precipitated solid was extracted into ether (500 ml). The ether solution was washed with water and brine, dried over magnesium sulfate, filtered, and treated with *N,N*-dicyclohexylamine (12 ml, 60 mmol). After cooling, a white solid was collected to yield 18.4 g (77%) of product, mp 157–158°, $[\alpha]^{25}D$ −17.3° (c 1, DMF).

Anal. Calcd for $C_{36}H_{53}N_3O_5S$: C, 67.56; H, 8.35; N, 6.57; S, 5.01. Found: C, 67.45; H, 8.40; N, 6.44; S, 5.03.

S-Benzhydryl-L-cysteinyl-L-alanine-½-Trifluoroacetate. The above salt (9 g, 0.014 mol) was neutralized with 1 *N* sulfuric acid. The resultant foam was dissolved in 40 ml of chloroform and treated with 40 ml of trifluoroacetic acid. The solution was stirred for 30 min and evaporated in vacuo. A solution of the residue in chloroform was treated with a mixture of ether–petroleum ether (1:1 v/v). The precipitate was collected by filtration, washed with ether, and dried in vacuo to give 5.1 g (80%) of product: mp 156–157° dec; $[\alpha]^{26}D$ −5.05° (c 0.99, DMF); homogeneous (system C).

Anal. Calcd for $C_{19}H_{22}N_2SO_3 \cdot \frac{1}{2}F_3C_2O_2H$: C, 57.05; H, 5.37; N, 6.63; S, 7.62; F, 6.77; residue, 1.2. Found: C, 56.91; H, 5.30; N, 6.55; S, 7.65; F, 6.74; residue, 1.14.

N-*tert*-Butyloxycarbonyl-S-trityl-L-cysteinylglycyl-N^{ϵ}-*tert*-butyloxycarbonyl-L-lysyl-L-phenylalanylglycyl-S-benzhydryl-L-cysteinyl-L-alanine (X). A solution of 5.86 g (5.96 mmol) of the pentapeptide derivative XVIII and 2.07 g (18 mmol) of *N*-hydroxysuccinimide in DMF (30 ml) at 10° was treated with 1.47 g (7.2 mmol) of DCC. The reaction mixture was allowed to stir for 2 hr at −10° and overnight at 0°. DCU was removed by filtration and the remaining solution was poured into ether (600 ml). The precipitate was collected by filtration, washed with hot 2-propanol (80 ml) twice, and dried in vacuo to give a white solid (4.3 g, 73%). The active ester was used without further purification.

The active ester (4.5 g, 4.3 mmol) was dissolved in 10 ml of DMF and added to a solution containing 3.7 g (7 mmol) of S-benzhydryl-L-cysteinyl-L-alanine-½-trifluoroacetate salt and 1.42 g (14 mmol) of *N*-methylmorpholine in 20 ml of DMF. The solution was stirred for 24 hr and the resulting precipitate was filtered, washed with ether, 1 *N* sulfuric acid, and water, and dried. The solid was dissolved in acetic acid and precipitated with water twice and once with ether. The compound was dissolved in 10 ml of DMF and applied to a silica gel (300 g) column poured with chloroform. Elution with chloroform–methanol–acetic acid (18:1:1 v/v) provided a white solid. The solid was recrystallized twice from methanol–ether to provide the pure heptapeptide derivative, X (2.8 g, 51%): mp 180–181.5° dec; $[\alpha]^{25}D$ −17.60° (c 0.5, DMF); homogeneous (system D).

Anal. Calcd for $C_{70}H_{84}N_8S_2O_{12}$: C, 64.97; H, 6.54; N, 8.66; S, 4.96. Found: C, 64.73; H, 6.64; N, 8.50; S, 4.89.

Amino acid analysis of a performic acid oxidized acid hydrolysate gave $CySO_3H$, 1.68; Gly, 2; Ala, 1.1; Phe, 0.98; Lys, 1.1.

N-*tert*-Butyloxycarbonyl-S-benzyhydryl-L-cysteinylglycyl-N^{ϵ}-*tert*-butyloxycarbonyl-L-lysyl-L-phenylalanylglycyl-L-cysteinyl-L-alanine (XIX) was prepared by treatment of a solution of IX (1.56 g, 1.2 mmol) in DMF (10 ml) and methanol (5 ml), contained in a round-bottom flask wrapped in aluminum foil, with a solution of silver nitrate (0.61 g, 3.6 mmol) and pyridine (0.3 ml, 36 mmol) in methanol (36 ml). The reaction mixture was stirred for 4 hr. Addition of ether (500 ml) to the reaction mixture precipitated a gelatinous solid which was collected by filtration, washed with ether, and dried. Mercaptoethanol (1.3 ml, fivefold excess) was added to a suspension of the silver mercaptide in 1:1 methanol–DMF (30 ml). The reaction mixture was stirred for 1 hr. The yellow precipitate was removed by filtration and the filtrate was poured into deoxygenated water (500 ml). The precipitate was collected, washed, and dried to yield 1.15 g (92%) of solid: mp 175–177° dec; $[\alpha]^{26}D$ −15.8° (c 0.5, DMF); homogeneous (system C, D).

Anal. Calcd for $C_{51}H_{70}N_8O_{12}S_2$: C, 58.44; H, 6.73; N, 10.41; S, 6.11. Found: C, 58.32; H, 6.92; N, 10.34; S, 6.11.

N-*tert*-Butyloxycarbonyl-L-cysteinylglycyl-N^{ϵ}-*tert*-butyloxycarbonyl-L-lysyl-L-phenylalanylglycyl-S-benzhydryl-L-cysteinyl-L-alanine (XXI) was prepared by treatment of a solution of 129.4 mg (0.1 mmol) of X in 2 ml of DMF with a solution of silver nitrate (51 mg, 0.3 mmol) and pyridine (0.03 ml, 0.3 mmol) in methanol (2 ml) in the dark. The reaction mixture was stirred for 4 hr and ether (50 ml) was added. The precipitate was collected and dried. The silver mercaptide in DMF (2 ml) was treated with mercaptoethanol (0.1 ml, fivefold excess) and stirred for 1 hr. The reaction mixture was filtered and the filtrate was poured into water (50 ml). The precipitate was collected and washed with ether. Recrystallization from methanol–ether gave a white solid (84 mg, 84%): mp 184–186° dec; $[\alpha]^{27}D$ −29.4° (c 0.5, DMF); homogeneous (system C, D).

Anal. Calcd for $C_{51}H_{70}N_8O_{12}S_2$: C, 58.44; H, 6.73; N, 10.41; S, 6.11. Found: C, 58.18, H, 6.82; N, 10.45; S, 5.95.

S,S-Bis(N-*tert*-butyloxycarbonyl-S-benzhydryl-L-cysteinylglycyl-N^{ϵ}-*tert*-butyloxycarbonyl-L-lysyl-L-phenylalanylglycyl-L-hemicystyl-L-alanine) (XX). A solution of 800 mg (0.38 mmol) of XIX in 20 ml of a DMF–methanol (1:1 v/v) mixture was titrated with 0.1 *N* iodine in methanol until a yellow color persisted. After stirring for 10 min, 2 drops of a 0.1% aqueous solution of sodium thiosulfate solution was added and the reaction mixture was poured into 500 ml of water. The precipitate was collected by filtration, dried in vacuo, and washed with ether to give 780 mg (98%) of the disulfide: mp 193–194° dec; $[\alpha]^{25}D$ −34.0° (c 0.5, DMF); homogeneous (system C, D).

Anal. Calcd for $C_{102}H_{138}N_{16}O_{24}S_4$: C, 58.33; H, 6.62; N, 10.67; S, 6.11. Found: C, 58.43; H, 6.65; N, 10.83; S, 6.25.

Preparation of Thiocyanogen Solution. The reagent was prepared by modification of the method of Wood.[14] All glassware was dried at 110° prior to use. The bromine was weighed into ethyl acetate or chloroform to give a solution of the appropriate concentration. The desired amount of bromine solution was added to a 25% excess of lead thiocyanate suspended in ethyl acetate or chloroform in a round-bottom flask wrapped in aluminum foil equipped with a calcium chloride drying tube. The reaction mixture was stirred until the bromine color disappeared. The colorless solution was taken with a pipet equipped with an inverted sinter funnel. This reagent was always prepared immediately before use.

***N*-*tert*-Butyloxycarbonyl-*S*-benzhydryl-L-cysteinylglycyl-N^{ϵ}-*tert*-butyloxycarbonyl-L-lysyl-L-phenylalanylglycyl-*S*-(*N*-*tert*-butyloxycarbonyl-L-cysteinylglycyl-N^{ϵ}-*tert*-butyloxycarbonyl-L-lysyl-L-phenylalanylglycyl-*S*-benzhydryl-L-cysteinyl-L-alanine)cysteinyl-L-alanine (XXIV).** A solution of XIX (262.8 mg, 0.25 mmol) in glacial acetic acid (40 ml) and chloroform (20 ml) was added dropwise to thiocyanogen solution (6.0 ml, 0.3 mmol) in chloroform at 0° in the dark. The reaction mixture was stirred for 30 min at 0°. A solution of 323.4 mg (0.25 mmol) of the *S*-trityl heptapeptide X in 10 ml of acetic acid was added to the reaction mixture and stirred for 27 hr at 0° in the dark. Chloroform was removed by evaporation and the remaining solution was poured into water (500 ml). The precipitate was coagulated by adding solid sodium chloride, collected by filtration, and dried in vacuo. The crude product was washed with ether, ethyl acetate, and chloroform. Two recrystallizations from methanol–ethyl acetate provided a pure compound XXIV (360 mg, 69%): mp 185–186° dec; $[\alpha]^{25}D$ −54.08° (*c* 0.49, DMF); homogeneous (system E).

Anal. Calcd for $C_{102}H_{138}N_{16}O_{24}S_4$: C, 58.33; H, 6.62; N, 10.67; S, 6.11. Found: C, 58.06; H, 6.81; N, 10.47; S, 5.98.

***S,S*-*S'*,*S'*-Bis(L-hemicystylglycyl-L-lysyl-L-phenylalanylglycyl-L-hemicystyl-L-alanine) (III).** A solution of XX (158 mg, 0.075 mmol) in 150 ml of trifluoroacetic acid–acetic acid (1:3 v/v) was treated with 1.9 ml (0.094 mmol, 25% excess) of a thiocyanogen solution in ethyl acetate at 0° in the dark and stirred for 48 hr. The reaction was lyophilized to a pink, fluffy powder which was treated with trifluoroacetic acid (5 ml) at 0° and stirred for 2 hr. The product was precipitated with ether and collected by filtration. The solid was dissolved in acetic acid and precipitated with ether. The crude product (110 mg) showed one spot (system I) and a lower faint streak.

A 1.5 × 60 cm column of Bio-Rex 70 (200–400 mesh) was used for purification. The column was freshly prepared before each run and equilibrated with 1% (v/v) aqueous acetic acid. The sample was dissolved in 1% acetic acid (1 ml) and applied to the column. A linear gradient of acetic acid (from 1% to 80%) was used for elution with a flow rate of 20 ml/hr. Fractions (3 ml) were collected in each tube. The effluent was examined by the Folin–Phenol reagent and monitored by absorbancy at 700 nm. Appropriate fractions were pooled and lyophilized.

The first peak was collected and lyophilized to give a white, fluffy powder (12.3 mg). TLC and paper electrophoretic mobilities of the material were identical with those exhibited by the material in the second peak (III). The electrophoretic pattern of the trypsin digest of the material was also the same as the pattern exhibited by III.

Anal. Calcd for $C_{56}H_{84}N_{16}O_{16}S_4 \cdot 2C_2F_3OOH \cdot Ca(C_2F_3O_2)_2 \cdot 4H_2O$: C, 39.72; H, 4.89; N, 11.59; S, 6.70; F, 11.70; residue calculated as CaO, 2.84; Ca^{2+}, 2.01. Found: C, 38.32; H, 4.66; N, 11.30, S, 7.52; F, 11.13; residue, 3.18; Ca^{2+}, 2.25.

Amino acid analysis of a performic acid oxidized acid hydrolysate gave $CySO_3H_{1.87}$, $Gly_{2.0}$, $Phe_{0.98}$, $Ala_{0.97}$, $Lys_{0.94}$.

The second peak was collected and lyophilized to give a white powder, III (58 mg, 57%): mp 160–169° dec; $[\alpha]^{25}D$ −63.26° (*c* 0.49, H_2O); homogeneous (system I); single spot on paper electrophoresis.

Anal. Calcd for $C_{56}H_{84}N_{16}O_{16}S_4 \cdot 4H_2O$: C, 46.60; H, 6.53; N, 15.40; S, 8.90. Found: C, 46.02; H, 6.50; N, 14.93; S, 8.63.

Amino acid analysis of a performic acid oxidized acid hydrolysate gave $CySO_3H_{2.0}$, $Gly_{2.04}$, $Ala_{1.0}$, $Phe_{0.92}$, $Lys_{1.0}$.

***S,S'*-L-Hemicystylglycyl-L-lysyl-L-phenylalanylglycyl-L-hemicystyl-L-alanine (V).** A solution of XIX (250 mg, 0.25 mmol) in acetic acid (20 ml) was added dropwise to a thiocyanogen solution (10 ml, 3.1 mmol, 25% excess) in ethyl acetate for 10 min at 0° in the dark and stirred for 20 min. The reaction mixture was diluted with 330 ml of trifluoroacetic acid–acetic acid (1:3 v/v) and stirred for 48 hr at 0° in the dark. Lyophilization gave pink, fluffy powder which was treated with 5 ml of trifluoroacetic acid at 0° and stirred for 2 hr. The product was precipitated with cold ether and collected. The solid was dissolved in water and filtered, and the remaining solution was lyophilized to a solid (140 mg) which exhibited one spot on TLC (system I) and a faint streak below the major spot. Purification of the product was carried out using chromatography on Bio-Rex 70 resin with a linear gradient of acetic acid (1% to 80%). Appropriate fractions were pooled and lyophilized to give a white, fluffy powder, V (100 mg, 59%): mp 173–182° dec; $[\alpha]^{25}D$ −16.00° (*c* 0.5, H_2O); homogeneous (system I); single spot on paper electrophoresis.

Anal. Calcd for $C_{28}H_{42}N_8O_8S_2 \cdot CH_3COOH \cdot 2H_2O$: C, 46.33; H, 6.40; N, 14.25; S, 8.25. Found: C, 46.69; H, 6.04; N, 13.90; S, 8.30.

Amino acid analysis of a performic acid oxidized acid hydrolysate gave $CySO_3H_{2.0}$, $Gly_{2.1}$, $Ala_{0.94}$, $Phe_{0.93}$, $Lys_{1.0}$.

***S,S'''*-L-Hemicystylglycyl-L-lysyl-L-phenylalanylglycyl-*S''*-(L-cysteinyl-glycyl-L-lysyl-L-phenylalanylglycyl-L-hemicystyl-L-alanine)-L-cysteinyl-L-alanine (IV).** A solution of 180 mg (0.086 mmol) of XXIV in 200 ml of trifluoroacetic acid–acetic acid (1:3 v/v) was treated with a thiocyanogen solution (2.16 ml, 0.108 mmol, 25% excess) in ethyl acetate at 0° in the dark and stirred for 48 hr. The reaction mixture was lyophilized to a pink, fluffy powder which was treated with trifluoroacetic acid (5 ml) at 0° and stirred for 2 hr. The product was precipitated with ether and collected by filtration. The solid was dissolved in water and filtered, and the remaining solution was lyophilized to a solid (130 mg) which exhibited one spot on TLC (system I) with a slight streak; no loop disulfide (V) was detected. Final purification was carried out using chromatography on Bio-Rex 70 resin with linear gradient of acetic acid (1% to 80%). Appropriate fractions were pooled and lyophilized to give a fluffy powder (15.5 mg) which exhibited mobilities on TLC and paper electrophoresis and an electrophoretic pattern of the tryptic digest identical with the second peak.

Anal. Calcd for $C_{56}H_{84}N_{16}O_{16}S_4 \cdot 2C_2F_3OOH \cdot Ca(C_2F_3OO)_2 \cdot 4H_2O$: C, 39.72; H, 4.89; N, 11.59; S, 6.70; F, 11.70; residue calculated as CaO, 2.84; Ca^{2+}, 2.01. Found: C, 38.02; H, 4.48; N, 10.90; S, 6.98; F, 11.71; residue 2.37; Ca^{2+}, 1.70.

Amino acid analysis of a performic acid oxidized acid hydrolysate gave $CySO_3H_{1.95}$, $Gly_{2.0}$, $Ala_{1.0}$, $Phe_{0.84}$, $Lys_{0.91}$.

The eluent of the second peak was collected and lyophilized to give a fluffy, white powder, IV (85 mg, 75%): mp 178–186° dec; $[\alpha]^{25}D$ −46.4° (*c* 0.5, H_2O); homogeneous (system I); one spot on paper electrophoresis.

Anal. Calcd for $C_{56}H_{84}N_{16}S_4O_{16} \cdot 2CH_3COOH$: C, 48.45; H, 6.25; N, 15.07; S, 8.70. Found: C, 48.09; H, 6.38; N, 14.86; S, 9.08.

Amino acid analysis of a performic acid oxidized acid hydrolysate gave $CySO_3H_{2.0}$, $Gly_{2.04}$, $Ala_{1.0}$, $Phe_{0.92}$, $Lys_{1.0}$.

Trypsin Digest Studies. A. Preparation of Enzyme Reagent. A 0.2% (w/v) solution of the enzyme reagent was prepared by dissolving trypsin (10 mg) in hydrochloric acid (5 ml, 0.001 *N*).[14]

B. Preparation of Substrate. A 0.5% (w/v) solution of the substrate was prepared by dissolving the cystine peptide (2 mg) in buffer solution (0.4 ml).[15] Enzyme digestion of the substrate was performed at pH 7.1 (0.2 *M*, sodium phosphate buffer solution). pH 6.3 (0.2 *M*, sodium phosphate buffer solution), and pH 8.6 (1 *M*, ammonium bicarbonate).

C. Hydrolytic Digestion. The enzyme reagent (0.05 ml, 0.1 mg of trypsin) was added to the substrate solution (2 mg in 0.4 ml of buffer solution) at room temperature and the reaction was allowed to proceed for 2 hr.

1. Bis Dimer III. Hydrolysis of III was performed at both pH 7.1 and 6.3. Two spots were detected at both reaction mixtures on paper electrophoresis. Spot 1, VI, migrated 40 cm (80 min); spot 2, VII, migrated 18 cm (80 min). Hydrolysis was complete after 5 min at pH 7.1. [The bis dimer III migrated 32 cm (80 min).]

2. Bis Dimer IV. Hydrolysis of IV was performed at both pH 7.1 and 6.3. One spot, VIII, detected on paper electrophoresis, migrated 29 cm (80 min) [bis dimer IV showed one spot, migrated 32 cm (80 min)].

3. Monomer V. Hydrolysis of V was performed at pH 7.1 for 2 hr. On paper electrophoresis, no change of V was detected. On prolonged digestion, a new minor spot, VIII [migrated 29 cm (80 min)], was shown above the major spot, V [migrated 28 cm (80 min)]. Two faint spots, 1, VI [migrated 40 cm (80 min)] and 2, VII [migrated 18 cm (80 min)], were also shown. When hydrolysis of V was performed at pH 8.6, in addition to the major spot [migrated at 28 cm (80 min)], three more minor spots were observed [migrated 29, 18, 40 cm (80 min)].

D. Performic Acid Oxidation. Performic acid was prepared by

mixing one part of 30% hydrogen peroxide and nine parts of formic acid (v/v) and allowing the mixture to stand at room temperature for 1 hr before use.[16]

1. Oxidation of Cystine Peptides. The cystine peptide (2 mg) was dissolved in performic acid (0.1 ml) and kept at 0° for 1 hr. The solution was diluted with water (10 ml) and lyophilized. On paper electrophoresis, the oxidative products of III, IV, and V all exhibited one spot, XXVII, migrated 3 cm (80 min).

2. Oxidation of Enzymic Hydrolysis Products. The digested samples of III and IV were acidified to pH 2.2 with hydrochloric acid (1 *N*), diluted with water, and lyophilized. The residues were dissolved in performic acid (0.1 ml) and kept at 0° for 1 hr. The solution was diluted with water (10 ml) and lyophilized. On paper electrophoresis, both of the samples of the oxidized tryptic digests of III and IV exhibited two spots. Spot 1, XXV, migrated 15 cm (80 min). Spot 2, XXVI, remained at the origin.

E. Isolation of Trypsin Hydrolysis Products of III. The digested sample III was acidified to pH 2.2 with hydrochloric acid (1 *N*), diluted with water, and lyophilized. The residue was dissolved in water (0.1 ml) and applied to Whatman no. 3 MM paper (46 × 57 cm) as a narrow band (3 cm). Electrophoresis was performed as described above. Spot visualization was achieved by cutting a strip from the edge of the narrow band on the dried paper, spraying with ninhydrin reagent, and drying. Comparison with the ninhydrin test strip allowed the isolation of each spot by cutting the area corresponding to the spot from the paper. Descending chromatography with aqueous acetic acid (5%, 20 ml) provided a solution of each compound which was lyophilized and assayed by amino acid analysis.

Amino Acid Analysis. Spot 1, VI [migrated 40 cm (80 min)]. Calcd: $CysSO_3H$, 1.0; Gly, 1.0; Lys, 1.0. Found: $CysSO_3H$, 1.02; Gly, 1.0; Lys, 1.01.

Spot 2, VII [migrated 18 cm (80 min)]. Calcd: $CysSO_3H$, 1.0; Gly, 1.0; Phe, 1.0; Ala, 1.0. Found: $CysSO_3H$, 0.97; Gly, 1.0; Phe, 0.93; Ala, 0.94; Lys, 0.

Chymotrypsin Digest Studies. A. Preparation of Enzyme Reagent. A 0.3% (w/v) solution of the enzyme reagent was prepared by dissolving α-chymotrypsin (3 mg) in hydrochloric acid (1 ml, 0.001 N).[15]

B. Preparation of Substrate. The preparation followed the procedure used on trypsin digest studies.

C. Hydrolytic Digestion. The enzyme reagent (0.015 ml, 0.04 mg of chymotrypsin) was added to the substrate solution (2 mg of substrate in 0.04 ml of buffer solution) at room temperature and the reaction was allowed to proceed for 2 hr.

1. Bis Dimer III. Hydrolysis of III was performed at pH 7.1. On paper electrophoresis, the hydrolytic sample exhibited two spots. Spot 1, XXVIII, migrated 34 cm (80 min); spot 2, XXIX, migrated 20 cm (80 min).

2. Bis Dimer IV. Hydrolysis of IV was performed at pH 7.1. On paper electrophoresis, the hydrolytic sample exhibited one spot, XXX, migrated 29 cm (80 min) [bis dimer IV showed one spot, migrated 32 cm (80 min)].

3. Monomer V. Hydrolysis of V was performed at pH 7.1 for 2 hr. On paper electrophoresis, no change of V was observed. When hydrolysis of V was carried out at pH 8.6, a minor new spot, XXX [migrated 29 cm (80 min)], above the major spot, V [migrated 28 cm (80 min)], was observed Possibly two other spots, XXVIII [migrated 34 cm (80 min)] and XXIX [migrated 20 cm (80 min)], were visible, though the spots were very faint.

D. Oxidation of Enzyme Hydrolysis Products. The reaction followed the procedure used for the trypsin digest studies. The oxidative products of the digested sample of III and IV exhibited two spots on paper electrophoresis. Spot 1 (oxidation of XXVIII) migrated 14 cm (80 min); spot 2 (oxidation of XXIX) showed very faint color at origin.

E. Isolation of Chymotrypsin Hydrolysis Products of III. The experimental procedure followed the method used for the trypsin digest studies.

Amino Acid Analysis. Spot 1, XXVIII, migrated 34 cm (80 min). Calcd: $CysSO_3H$, 1; Gly, 1; Lys, 1; Phe, 1. Found: $CysSO_3H$, 0.81; Gly, 1.0; Lys, 0.94; Phe, 1.11. Spot 2, XXIX, migrated 20 cm (80 min). Calcd: $CysSO_3H$, 1; Gly, 1; Ala, 1. Found: $CysSO_3H$, 0.89; Gly, 1.0; Ala, 0.88; Lys, 0.

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Registry No.—III, 56678-69-4; IV, 56689-35-1; V, 56689-37-3; IX, 56678-70-7; X, 56678-71-8; XI, 33515-73-0; XII, 56678-73-0; XIII, 56678-74-1; XIV, 56678-75-2; XV, 56678-76-3; XVI, 56678-77-4; XVII, 56678-78-5; XVIII, 56678-79-6; XIX, 56678-80-9; XX, 56678-81-0; XXI, 56678-82-1; XXIV, 56678-83-2; N^{ϵ}-*tert*-butyloxycarbonyl-L-lysine, 2418-95-3; L-phenylalanylglycine, 721-90-4; *N*-*tert*-butyloxycarbonyl-*S*-trityl-L-cysteine *N*,*N*-dicyclohexylammonium salt, 26988-59-0; L-alanine, 56-41-7; N^{α}-carbobenzyloxy-N^{ϵ}-*tert*-butyloxycarbonyl-L-lysine *N*-hydroxysuccinimide ester, 3338-34-9; N^{ϵ}-*tert*-butyloxycarbonyl-L-lysyl-L-phenylalanylglycine, 56678-84-3; *N*-*tert*-butyloxycarbonyl-*S*-benzhydryl-L-cysteinyl-L-alanine *N*,*N*-dicyclohexylammonium salt, 56678-75-2; *N*-*tert*-butyloxycarbonyl-*S*-benzhydryl-L-cysteine *N*,*N*-dicyclohexylammonium salt, 26988-51-2; *S*-benzyhydryl-L-cysteinyl-L-alanine–½-trifluoroacetate, 56678-86-5.

References and Notes

(1) For the previous paper in this series see R. G. Hiskey, N. Muthukumaraswamy, and R. R. Vunnam, *J. Org. Chem.*, **40**, 950 (1975).
(2) Abstracted in part from a dissertation by C. Li submitted in partial fulfillment of the requirements for the Ph.D., University of North Carolina at Chapel Hill, 1973.
(3) The following abbreviations have been employed in the text: BOC = *tert*-butyloxycarbonyl; Bzh = benzhydryl; Tr = trityl; DCHA = *N*,*N*-dicyclohexylamine; DCC = *N*,*N*′-dicyclohexylcarbodiimide; DCU = *N*,*N*′-dicyclohexylurea; HONSu = *N*-hydroxysuccinimide; NMN = *N*-methylmorpholine; BME = β-mercaptoethanol; DME = 1,2-dimethoxyethane; DMF = *N*,*N*-dimethylformamide; DMAc = *N*,*N*-dimethylacetamide.
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